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(54) Title: AMINO ACID DEGRADING ENZYMES MODULATE CELL DEATH

(57) Abstract

Under some conditions which normally induce cell death, the addition of amino acid degrading enzymes protect cells from death. Under other conditions, such as TNFα exposure, amino acid degrading enzymes potentiate TNFα–induced cell death. Both of these amino acid degrading enzyme actions may result from inhibition of protein synthesis. Screening can identify compounds that affect the amino acid degrading enzyme–mediated protection. These compounds, as well as the amino acid degrading enzymes themselves, may be used for the treatment of diseases associated with cell death. In addition, amino acid degrading enzymes may be used in conjunction with TNFα to increase cancer cell death.

AMINO ACID DEGRADING ENZYMES MODULATE
CELL DEATH

Background of the Invention

This invention relates to the modulation of cell death.

The discovery that cells undergo programmed cell death, or apoptosis, has

5   generated considerable scientific interest. It has been demonstrated that programmed

cell death plays a crucial role in a number of normal physiological processes,

including morphological development, clonal selection in the immune system, and

normal cell maturation and death in other tissue and organ systems.

If the regulation of cell death is abnormal, the effect on cell survival has

10   several important implications related to the etiology and treatment of certain diseases.

First, abnormal regulation may result in either premature cell death or tumorigenesis.

Second, if the abnormal cell death is dependent upon a known mechanism, it may be

possible to suppress this mechanism as a means of treating disease.

Premature death of neurons by a degenerative process can have particularly

15   severe ramifications, given that mature neuronal cells are thought to be incapable of

cell division. Neurodegeneration is a major pathological feature of many human

neurological disorders including traumatic injury, ischemia, and chronic diseases such

as: Alzheimer's disease (Gshwind et al., J. Neurochem. 65:292-300, 1995),

Huntington's disease (Portera-Cailliau et al., J. Neurosci. 15:3775-3787, 1995),

20   Parkinson's Disease (Ozawa et al., Biochem. Biophys. Res. Commun. 235:158-61,

1997) spinal muscular dystrophy (Roy et al., Cell 80:165-178, 1995), amyotrophic

lateral sclerosis (ALS) (Alexianu et al., J. Neurochem. 63:2365-2368, 1994), stroke

(Linnik et al., Stroke 24: 2002-2004, 1993) and spinal cord injury (Crowe et al.,

Nature Med. 3:73-76, 1997).

25   Among the many paradigms used to induce premature cell death, attention has

been directed to oxidative stress (Ratan et al., J. Neurochem. 62:376-379, 1994; Ratan

et al., J. Neurosci. 14: 4385-4392, 1994; Halliwell, Ann Neurol 32 (Suppl.), S10-S15,

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1992), and viral infection (Levine et al., Nature (London) 361:739-742, 1993; Gougeon and Montagnier, Science (Wash. DC) 253:401-05, 1993; Pahl and Baeuerle, J. Virol. 69:1480-84, 1993). These two paradigms apparently function by different pathways. Although antioxidants can reverse cell death associated with oxidative stress (Murphy et al., Dev. Brain Res. 57:146-50, 1990), antioxidants do not reverse cell death associated with Sindbis virus infection in cortical neurons or neuronal cell lines (Lin et al., J. of Cell Biology 131:1149-61, 1995).

While suppression of cell death may be effective in treating certain diseases, other diseases, such as cancer, may be effectively treated by increasing cell death in specific cells. Tumor necrosis factor (TNF) was initially named for its ability to shrink tumors, and was promising as an anti-cancer treatment. However, TNF is not effective in killing many types of cancer cells (Barinaga, Science 274: 724, 1996).

Summary of the Invention

In a first aspect, the invention features a method for determining whether a chemical compound affects an amino acid degrading enzyme-mediated protection against nonexcitotoxic, NO-independent cell death that includes contacting mammalian cells with the compound in the presence of an exogenous amino acid degrading enzyme which protects the cells against cell death, in the absence of excitotoxic stimulation and NO formation, and further includes determining the effect of the compound on the amino acid degrading enzyme-mediated protection against cell death.

In one preferred embodiment of the first aspect of the invention, the mammalian cells are neuronal cells. In another embodiment of this aspect, the mammalian cells are glial cells. In another embodiment, the mammalian cells are immortalized mouse fibroblast cells. In another preferred embodiment, the exogenous amino acid degrading enzyme is arginase. In another embodiment, the exogenous amino acid degrading enzyme is arginine decarboxylase. In another embodiment, the

exogenous amino acid degrading enzyme is arginine deiminase. In another

embodiment, the exogenous amino acid degrading enzyme is asparaginase. In yet

another embodiment, the exogenous amino acid degrading enzyme is expressed in the

mammalian cells from a recombinant DNA molecule encoding the amino acid

5   sequence of the amino acid degrading enzyme.

In a second aspect, the invention features a method for determining whether a

chemical compound affects an amino acid degrading enzyme-mediated protection

against nonexcitotoxic, NO-independent cell death that includes contacting the

compound with an amino acid degrading enzyme which protects the cells against cell

10 death, and determining the effect of the compound on the activity of the enzyme.

In a third aspect, the invention features a method for determining whether a

chemical compound affects amino acid degrading enzyme protection against

nonexcitotoxic, NO-independent cell death in a mammalian nervous system that

includes identifying a compound that affects the protection against cell death, by the

15 method of the first aspect of the invention, contacting the central nervous system of a

mammal with the compound, in the absence of excitotoxic stimulation or NO

formation, and determining the effect of the compound on the protection against cell

death in the central nervous system of the mammal.

In a fourth aspect, the invention features a method for inhibiting

20 nonexcitotoxic, NO-independent cell death in the central nervous system of a mammal

that includes administering to the mammal a cell death reducing amount of an amino

acid degrading enzyme.

In a preferred embodiment of the fourth aspect of the invention, the amino acid

degrading enzyme is human arginase.

25 In a fifth aspect, the invention features a method for treating a human cancer

patient that includes administering to the patient TNFα together with an amount of an

amino acid degrading enzyme that is sufficient to potentiate TNFα-induced cell death.

In a preferred embodiment of the fifth aspect, the amino acid degrading enzyme

is human arginase.

By "amino acid degrading enzyme" is meant an enzyme which splits one or
more chemical groups from an amino acid substrate.

By "protection against cell death" is meant an action that inhibits cell death.

By "nonexcitotoxic, NO-independent mechanisms of cell death" is meant
mechanisms that cause death independent of excitation by excitatory amino acids or
the formation of nitric oxide (NO).

By "a recombinant DNA molecule" is meant a nucleic acid molecule or
polypeptide having been purified (i.e., separated by genetic, physical, or enzymatic
methods) from the naturally occurring sequences with which it is naturally linked.

By "expression" is meant the transcription and translation of a DNA molecule
resulting in the production of a polypeptide encoded by the nucleic acid sequence of
the molecule.   —

By "potentiating" is meant the interacting of a combination of compounds such
that the total effect of the combination is greater than the sum effect of each
compound acting alone.

Other features and advantages of the invention will be apparent from the
following detailed description thereof, and from the claims.

Brief Description of the Drawings

Figure 1 is a graph showing cell death in E18 cortical neurons subjected to
oxidative stress by glutathione depletion. Increasing concentrations of arginase in the
cell medium decreased neuronal death. The percent neuronal survival was derived by
comparison to neurons free of arginase and under no oxidative stress.

Figure 2 is a graph showing cell death in E18 cortical neurons infected with
Sindbis virus. Increasing concentrations of arginase in the cell medium decreased
neuronal death. The percent neuronal survival was derived by comparison to

uninfected neurons free of arginase.

Figure 2A is a graph showing cell death in N18 neuroblastoma cells infected with Sindbis virus. Increasing concentrations of arginase in the cell medium decreased neuronal death. The percent neuronal survival was derived by comparison to uninfected neuroblastoma cells.

Figure 3 is a bar graph showing cell death in E17 cortical neurons exposed to staurosporine (STS). Arginase (0.5 μg/ml) decreased neuronal death. The percent neuronal survival was derived by comparison to unexposed neurons.

Figure 4 is a graph showing cell death in N18 neuroblastoma cells infected with Sindbis virus. Increasing concentrations of arginine decarboxylase decreased cell death. The percent survival was derived by comparison to uninfected cells.

Figure 5 is a bar graph showing cell death in Sindbis virus infected N18 neuroblastoma cells. Exposure to asparaginase (1-5 U/ml) decreased cell death. The percent survival was derived by comparison to uninfected cells.

Figure 6 is a bar graph showing cell death in Sindbis virus infected N18 neuroblastoma cells. Arginase (2.5 μg/ml) decreased cell death. Both Sindbis virus-induced cell death and arginase-induced protection against cell death were unaffected by the nitric oxide synthase inhibitors N^G-methyl-L-arginine (L-NMA) or guanidinoethydisulfide (GED). The percent survival was derived by comparison to uninfected cells.

Figure 7 is a bar graph showing cell death in E17 cortical neurons subjected to oxidative stress. Oxidative stress increased cell death, which was unaffected by the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME). The percent survival was derived by comparison to neurons under no oxidative stress.

Figure 8 is a graph showing the correlation between arginase-induced inhibition of protein synthesis (measured as a decrease in ^{35}S methionine/cysteine incorporation)

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and arginase-mediated protection against cell death in Sindbis virus infected N18

neuroblastoma cells. The percent survival was derived by comparison to uninfected

cells.

Figure 9 is a graph showing the potentiation of TNFα-induced cell death in 3T3

5   mouse embryo fibroblasts by increasing concentrations of arginase (0-2000 ng/ml).

Neither TNFα nor arginase alone caused significant cell death.

Detailed Description of the Invention

In general, the invention is directed to modulating mammalian cell death under

10   conditions that induce cell death via nonexcitatory, nitric oxide (NO)-independent

mechanisms. The modulatory effect is achieved by increasing the degradation of an

amino acid via the action of an amino acid degrading enzyme. Under conditions that

induce cell death through nonexcitatory, NO-independent mechanisms, such as

oxidative stress, Sindbis virus infection, and administration of staurosporine

15   administration (a protein kinase inhibitor), the action of an amino acid degrading

enzyme protects cells against death. In contrast, when cell death is induced by TNFα

administration, the action of an amino acid degrading enzyme potentiates cell death.

Despite these seemingly incongruous effects on cell death, the modulatory action of

amino acid degrading enzymes, under all of the above conditions, is likely mediated

20   by inhibiting protein synthesis via the degradation of amino acids.

The invention provides screening methods for identification of candidate

compounds that enhance the protection against cell death by amino acid degrading

enzymes under conditions that induce cell death through nonexcitotoxic, NO-

independent mechanisms. In addition, the invention provides a method of

25   administering an amino acid degrading agent to the mammalian central nervous

system to protect against cell death mediated by nonexcitotoxic, NO-independent

mechanisms. According to the invention, both the newly identified agonists of amino

acid-degrading enzymes and the enzymes, themselves, can be used as therapeutics as

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follows: 1) to protect cells of the central nervous system against death in the treatment of neurological diseases or disturbances characterized by increased premature cell death mediated by nonexcitotoxic, NO-independent mechanisms; and 2) to potentiate cell death induced by TNFα in the course of TNFα anti-cancer treatment.

5   The present invention is based upon studies of the mechanisms of cell death. We found that a bovine liver extract protected neurons against death due to a neuroprotective factor that we isolated and identified as arginase. Addition of recombinant arginase to the neuronal culture media confirmed that arginase mediated a protective effect against neuronal death under the variety of nonexcitotoxic, NO-

10   independent cell death-inducing conditions, e.g., oxidative stress, Sindbis virus infection, and staurosporine administration. Arginine decarboxylase also protected neurons against death, indicating that the protective effect is likely mediated by depletion of arginine, rather than an increase in the arginase reaction products, ornithine and urea.

15   This protective effect is not due to NO formation (Figure 6), but can be attributed to amino acid depletion and the consequent suppression of protein synthesis (Figure 8). This assertion is supported by the following: 1) inhibition of nitric oxide synthase (NOS) has no effect on the amino acid degrading enzyme effect (Figure 6); 2) the rate of cell survival is directly proportional to the inhibition of protein synthesis

20   (Figure 8); 3) similar protective effects are demonstrated with multiple amino acid degrading enzymes that use different amino acid substrates, e.g. arginine, arginine and asparagine (Figures 1-5); 4) the amino acid degrading enzyme, arginase, potentiates TNFα-induced cell death (Figure 9), which is a type of cell death known to be potentiated by inhibitors of protein synthesis (Beg and Baltimore, Science 274:782-

25   784, 1996); and 5) both oxidative stress, via glutathione depletion, and staurosporine-induced cell death are known to be inhibited by the inhibition of protein synthesis (Ratan et al., J. Neurosci. 14: 4385-4392, 1994; Koh et al., Exp. Neurol. 135: 153-159, 1995).

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Arginase mediated a protective effect against cell death under a variety of culture
conditions designed to induce nonexcitotoxic, NO-independent cell death

Methods

Primary cell cultures from the cerebral cortex of fetal Sprague-Dawley rats
(day 17 or 18 of gestation) were obtained as previously described (Murphy et al.,
FASEB J. 4:1624-33, 1990). Mouse N18 neuroblastoma cells were grown as
previously described (Levine et al., Nature (Lond) 361:739-42, 1993). Immortalized
mouse embryo 3T3 fibroblasts were grown as previously described (Beg and
Baltimore, 1996, *supra*). Cells were grown in multi-well culture plates in Minimum
Essential Medium (MEM) (Gibco BRL, Rockville, MD) containing 10% fetal calf
serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 µg/ml). N18
cell media also contained Earle Salts and 10% Myoclon Super Plus FBC (Gibco BRL,
Rockville, MD).   —

Oxidative stress, via glutathione depletion, was induced one day after plating
by replacing the media with MEM containing 10% fetal calf serum, 2 mM L-
glutamine, penicillin (50 units/ml), streptomycin (50 µg/ml), and either low cystine (1
µM), or glutamate (10 mM), or homocysteate (1 mM).

Sindbis virus infection was induced one day after plating by replacing the
media with low serum (2% fetal calf serum) MEM containing Sindbis virus (strain
AR339) at a multiplicity of infection of 1-5 plaque forming units per cell. After a one
hour infection period, the low serum media was replaced with MEM containing 10%
fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50
µg/ml).

For staurosporine exposure (Molecular Probes, Eugene, OR), cells were rinsed
once with phosphate buffered saline (PBS) (37°C) and then switched to MEM
containing 5.5 g/l glucose, 10% fetal calf serum, 2 mM L-glutamine, 100 µM cystine,
and 100 nM staurosporine.

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Recombinant rat liver arginase was expressed and purified as described previously (Cavalli et al., Biochemistry 33: 10652-10657, 1994). At the same time the cell death-inducing conditions were imposed, amino acid degrading enzymes were added directly to tissue culture plates as follows: arginase (recombinant or Sigma, St.

5   Louis, MO) was added in concentrations ranging from 0 to 4000 ng/ml; arginine decarboxylase (Sigma) was added to tissue culture plates at concentrations ranging from 0 to 4000 µg/ml, and asparaginase (Sigma), associated with polyethylene glycol (PEG-asparaginase) was added at 1-5 U/ml.

Cell viability was assessed 24 hours following oxidative stress, 48 hours

10   following Sindbis virus infection, and 60 hours following staurosporine exposure. Cell cultures were incubated for 20 minutes with 5 µM calcein A/M (Molecular Probes, Eugene, Oregon) to stain live cells and 5 µM propidium iodide (Molecular Probes, Eugene, Oregon) to stain dead cells, rinsing once with warm PBS and examining by fluorescence microscopy. Alternatively, cell viability was assessed

15   using trypan blue exclusion (Levine et al., Nature (Lond.) 361:739-41, 1993), lactate dehydrogenase (LDH) release (Ratan et al., J. Neurochem. 62:376-379, 1994; Ratan et al., J. Neurosci. 14:4385-4392, 1994), ethidium homodimer (Ethid-1; Molecular Probes) staining, or DNA fragmentation (Ratan et al., J. Neurochem 62:376-79, 1994).

20   Statistics

Results are presented as mean ± standard error of the mean for three to five experiments. Experimental groups with multiple treatments were analyzed by analysis of variance.

Results

25   Arginase protected cells from death under oxidative stress by glutathione depletion (GD), Sindbis virus infection (SV), and staurosporine exposure (STS).

For example, as is shown in Figure 1, increasing concentrations of arginase decreased cell death in E18 cortical neurons subjected to oxidative stress. Oxidative

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stress in the absence of arginase increased cell death, reducing cell survival to approximately 35% of control, whereas survival increased to about 75% of control at the arginase concentration of 2 µg/ml.

Furthermore, as is shown in Figure 2, increasing concentrations of arginase resulted in decreased death in E18 cortical neurons infected with Sindbis virus. Sindbis virus infection in the absence of arginase increased cell death, reducing cell survival to approximately 50% of control, whereas the highest concentration of arginase tested (4 µg/ml) restored cell survival to 100% of control.

The effect of Sindbis virus infection was modified somewhat when N18 neuroblastoma cells were used, as shown in Figure 2A. Sindbis virus infection in the absence of arginase reduced cell survival to approximately 10% of control. This value is lower than that reported in Figure 2 for E18 cortical neurons, a discrepancy which likely results from the presence of glial cells in the E18 cortical neuron preparation. These glial cells are less susceptible to death, and thus increase the overall percentage of cell survival. Figure 2A also demonstrates that N18 neuroblastoma cell survival increased to about 90% of control at the arginase concentration of 2 µg/ml.

In addition, as is shown in Figure 3, arginase (0.5 µg/ml) decreased cell death in cortical neurons exposed to staurosporine. Staurosporine exposure increased cell death, reducing cell survival to approximately 30% of control, whereas addition of arginase restored cell survival to approximately 90% of control.

Additional evidence indicates that the above described protective effect of arginase is due to amino acid degradation rather than formation of the arginase reaction products ornithine and urea. First, degradation of arginine by another degrading enzyme, arginine decarboxylase, also decreased cell death. As shown in Figure 4, Sindbis virus infection, in the absence of arginine decarboxylase, increased cell death in N18 neuroblastoma cells, reducing cell survival to approximately 12% of control, whereas increasing doses of arginine decarboxylase increased cell survival, restoring it to approximately 100% of control at a dose of 1 mg/ml. The EC 50 for

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protection was approximately 300 µg/ml. Second, degradation of another amino acid, asparagine, also decreased cell death. As shown in Figure 5, Sindbis virus infection reduced N18 neuroblastoma cell survival to about 12% of control. Addition of asparaginase (1-5 U/ml) to Sindbis virus infected cells increased cell survival to about 65% of control.

Taken together, these results demonstrate that amino acid degradation protects cells from death induced by a variety of cell-death inducing conditions, e.g., oxidative stress, Sindbis virus infection, and staurosporine exposure.

Protection from cell death by amino acid degradation is nitric oxide independent

Although arginine is a precursor of nitric oxide (NO), the following experiments show that arginase's protection against cell death is independent of NO formation.

Methods

N18 neuroblastoma cells were treated with the general nitric oxide synthase (NOS) inhibitor, N^G-methyl-L-arginine (L-NMA, 100 µM, Calbiochem, La Jolla, CA) (Dawson et al. Proc. Natl. Acad. Sci. USA 88:6368-6371, 1991; Schmidt et al., J. Biol. Chem. 269:1674-1680, 1994), or the inducible NOS inhibitor, guanidinoethydisulfide (GED, 100 µM, Calbiochem). The cells were pre- or co-treated with the NOS inhibitors in conjunction with SV infection, with or without arginase treatment (2.5 µg/ml).

Cortical neurons were treated with the general NOS inhibitor, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 - 500 µM, Calbiochem) in conjunction with either oxidative stress or low doses of staurosporine.

Results

In each of these paradigms, NOS inhibitors were not toxic to control cultures. In addition, the NOS inhibitors had no effect on cell death induced by SV infection (Figure 6), oxidative stress (Figure 7), and staurosporine exposure. In addition, the

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NOS inhibitors had no effect on arginase-mediated protection from cell death under

conditions of SV infection (Figure 6). Therefore, cell death induced by the above

mentioned conditions, and protection from cell death mediated by arginase, are

independent of NO.

5

Arginase-mediated protection is correlated with inhibition of protein synthesis

Arginine, in addition to being a precursor for NO, is also utilized for protein

synthesis. The following results show that the degree of arginase-mediated protein

synthesis inhibition is correlated with arginase-mediated cell survival.

10   ## Methods

Radioactive labeling experiments using ^{35}S-cysteine/methionine incorporation

were performed using EasyTag express protein labeling mix (New England Nuclear,

Boston, Mass.) as described previously (Ratan et al., J. Neurosci. 14:4385-4392, 1994)

with the following modifications. N18 cells were plated into 6 well dishes at a density

15   of 5 x 10^4 cells/well. Prior to labeling, the media were changed and replaced with

media containing arginase (0 to 4000ng/well) for 4 hours. 2μCi of ^{35}S

cysteine/methionine was then added to each well for 4 hours. The labeling was

stopped by three rapid washes with 4 mls of ice-cold PBS supplemented with 1mM

CaCl$_2$. Immediately after the washes, the cells were lysed with 3% perchloric acid,

20   scraped and transferred to eppendorf tubes. The samples were spun at 12,000 RPM in

a microfuge at 4°C for 20 minutes, and the radioactivity of an aliquot of the

supernatant was determined by liquid scintillation counting as a measure of the acid-

soluble ^{35}S-cysteine/methionine. The acid-precipitable pellet containing the labeled,

newly synthesized protein, was washed and repelleted. The supernatant was discarded

25   and the pellet was dissolved in 0.1 M NaOH. The radioactivity in this NaOH solute

was measured and the protein determined by the bicinchoninic acid reagent method

(Pierce, Rockford, IL). In parallel, viability assays were performed as described

above.

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Results

As shown in Figure 8, the decrease in protein synthesis was associated with an increase in cell survival in N18 neuroblastoma cells infected with Sindbis virus.

Arginase potentiates TNFα-induced cell death

In support of protein synthesis inhibition as the mechanism by which arginase and other amino acid degrading enzymes prevent cell death, the following results demonstrate that arginase potentiates cell death induced by tumor necrosis factor (TNFα), a paradigm known to be potentiated by the inhibition of protein synthesis (Beg and Baltimore, 1996, *supra*).

Methods

Immortalized mouse embryo 3T3 fibroblasts were grown as described (Beg and Baltimore, 1996, *supra*) at a density of 2,000 cells/well in 96 well plates, allowed to adhere overnight and treated with 10 ng/ml mouse TNFα (Boehringer Mannheim, Indianaolis, IN), with or without recombinant arginase (100-5,000 ng/ml). Viability was assessed 24 hours later using MTT or LDH and the viability index described above.

Results

Neither TNFα nor arginase treatment alone significantly affected cell viability. However, as is shown in Figure 9, increasing concentrations of arginase, in the presence of 10 ng/ml TNFα, resulted in increased cell death. The EC 50 for arginase-enhanced death, decreasing cell survival to 50% of control was approximately 8 µg/ml.

These results provide evidence that arginase potentiates TNFα-mediated cell death. These results can be reconciled with the previous results that demonstrated protection from cell death by considering that both effects occur as a result of the inhibition of protein synthesis. Although such inhibition may generally protect cells

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from death under conditions such as trauma, stroke, and chronic neurodegenerative disease, the condition of TNFα stimulation is an exception. In this case, inhibition of protein synthesis may deprive the cell of its protective response to TNFα, thus enhancing cell death when cells are treated with both TNFα and an amino acid

5   degrading enzyme.

Identification of compounds that enhance amino acid degrading enzyme-mediated protection against cell death

As described herein, addition of an amino acid degrading enzyme to the cell

10   culture medium results in increased survival in cell preparations subjected to conditions which normally induce cell death mediated by nonexcitotoxic, NO-independent mechanisms. Given these results, screens for compounds that enhance the described protective effect of the amino acid degrading enzymes may be carried out to identify drugs and provide therapies to treat disorders in humans and other

15   mammals associated with cell death mediated by nonexcitotoxic, NO-independent mechanisms. In addition to the amino acid degrading enzymes previously described, the following enzymes would also be suitable: tryptophanase, histidase, leucine dehydrogenase, lysine decarboxylase, L-methionini-gamma lyase, and phenylalanine ammonia lyase (Sigma, St. Louis, MO). A number of exemplary assays now follow.

20

1) Screening candidate compounds for the ability to enhance amino acid degrading   enzyme-mediated protection against cell death

a) Cells

In the first step of the screening assay, a cell line that exhibits amino acid

25   degrading enzyme protection against cell death, e.g., a primary neuronal cell culture, an immortalized neuronal or glial cell line, or mouse 3T3 fibroblasts, is prepared for experimentation by plating in multi-well culture dishes. Cell death is induced by application of cell death-inducing conditions known to cause death through

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nonexcitotoxic, NO-independent mechanisms, e.g., oxidative stress, Sindbis virus

infection, and staurosporine exposure. Alternatively, the possible contribution of

excitatotoxic or NO-dependent mechanisms of cell death can be eliminated by

addition of excitatory amino acid receptor antagonists, e.g., (+)-5-methyl-10,11-

dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine (MK-801), or 6-cyano-7-

nitroqinoxaline-2,3-dione, or by the addition of NOS inhibitors, e.g., 7-nitroindazole,

N-nitro-L-arginine, or aminoguanidine. Parallel wells of cells remain in non-cell death

inducing conditions.

An amino acid degrading enzyme, e.g., arginase, arginine decarboxylase,

arginine deiminase, or asparaginase in final concentrations ranging from 1 ng/ml to

100 mg/ml, and one or more candidate compounds are added to the culture medium at

some time in relation to induction of cell death, e.g., prior to, at the same time, or after

induction of cell death. Parallel wells of cells remain free of amino acid degrading

enzyme, or free of candidate compound, or free of both amino acid degrading enzyme

and candidate compound.

b) Reagents

The screening methods of the invention can employ any mammalian or

microbial form of amino acid degrading enzyme, e.g., arginase (Sigma, St. Louis,

MO), arginine decarboxylase (Sigma), arginine deiminase (recombinant expression;

for arginine deiminase sequence, see Kondo et al., Mol. Gen. Genet. 221: 81-86, 1990,

Harasawa et al., Microbiol. Immunol. 36: 661-665, 1992), or asparaginase (Sigma),

isolated from a natural source or made by recombinant expression (Cavalli et al.,

Biochemistry 33: 10652-10657, 1994). The preferred amino acid degrading enzymes

are those having the human amino acid arginase sequence.

Compounds to be screened, termed "candidate compounds," are preferably

soluble in aqueous solution, and may be proteinaceous, peptidyl, or non-peptidyl.

Candidate compounds can be individually or combinatorially added to each well.

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c) Output

Cell death is determined after varying times of incubation depending on the
conditions used for inducing cell death, e.g., optimal incubation is for 24 hours after
serum deprivation or oxidative stress, and 48 hours after Sindbus virus infection. Cell

5   death is assessed by measuring cell viability, e.g., by live and dead cell staining, LDH
release, DNA fragmentation, 3-4,5-dimethylthiozol-2-yl)-2,5 diphenyl tetrazolium
bromide (MTT) reduction (Gieni et al., 1996), or any other method used to assess cell
viability.

Cell number in the sample of control cells, cultured in non-cell death-inducing

10   conditions, free of added amino acid degrading enzyme and candidate compounds, is
established as 100 percent cell survival. Cell number in all of the samples of cells
subjected to cell death-inducing conditions with various combinations of arginine
depleting enzyme and candidate compounds is expressed as a percent cell survival
relative to the above described control.   —

15   d) Results

The candidate compound may enhance the amino acid degrading enzyme-
mediated protection against cell death if samples exposed to cell death-inducing
conditions, an amino acid degrading enzyme, and the candidate compound, have
greater cell survival than samples exposed to the same conditions, except excluding

20   the candidate compound.

If a candidate compound increases cell survival by specifically enhancing the
action of the amino acid degrading enzyme, then the sample exposed to both cell
death-inducing conditions and candidate compound, but no amino acid degrading
enzyme, will exhibit no enhanced cell survival, as compared to samples exposed only

25   to cell death-inducing conditions.

The candidate compound may protect cells against death by a pathway
independent of an amino acid degrading enzyme, if cell survival is also enhanced in
the sample exposed to cell death-inducing conditions, and the candidate compound,

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but no amino acid degrading enzyme, as compared to the sample exposed only to cell death-inducing conditions.

One will typically desire to include in the admixture an amount of amino acid degrading enzyme sufficient to produce a submaximal level of protection against death, e.g. 0.1-400 ng/ml, such that the protective effect of the enzyme can be consistently detected when compared to the effect seen in cells cultured in the absence of enzyme, but which also allows for detection of an enhanced effect produced by addition of the candidate substance over the effect of amino acid degrading enzyme alone.

e) Cells expressing an amino acid degrading enzyme

In another version of the screening assay, the method previously described is modified by using a cell line which is transfected with recombinant DNA that encodes an amino acid degrading enzyme, e.g., arginase, arginine decarboxylase, arginine deiminase, or asparaginase, resulting in expression of the enzyme in the cell. All steps of the assay are conducted as previously described, except the exogenous amino acid degrading enzyme is not directly added to the culture media, but, rather, is expressed by the cells themselves. Parallel wells of cells which do not express the enzyme are also assayed for comparison.

Under conditions inducing cell death, the amino acid degrading enzyme may enhance protection against cell death if survival in cells expressing the enzyme is greater than survival in cells which do not express the enzyme.

The candidate compound may enhance the amino acid degrading enzyme mediated protection against death if survival in cells expressing the enzyme exposed to cell death-inducing conditions and the candidate compound is greater than survival in the same type of cells exposed the same conditions, except excluding the candidate compound.

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2) Screening for compounds that enhance amino acid degrading enzyme activity

A preliminary method that assists in identifying compounds that enhance the amino acid degrading enzyme-mediated protection against apoptosis is screening candidate compounds for the ability to enhance the activity of the amino acid degrading enzyme.

Samples of arginine depleting enzyme, e.g., arginase, arginine decarboxylase, arginine deiminase, or asparaginase, are mixed with candidate compounds. Parallel samples of enzyme remain free of candidate compound. Amino acid degrading enzyme activity is then assayed, e.g., arginase activity is measured by determination of urea formation.

If the candidate compound enhances the enzymatic activity of the amino acid degrading enzyme, then the activity of the enzyme assayed in the presence of the candidate compound should increase compared to activity of the enzyme assayed in the absence of the candidate compound.

This assay can be used as a primary screen to identify compounds to be included in further screening for ability to enhance the amino acid degrading enzyme-mediated protection against cell death, as described herein.

3) Testing candidate compounds in animal models

a) Animal models

A number of animal models exist for the study of neurodegenerative disorders and find use in the screening methods described herein. For example, such models may serve as a system in which to screen candidate compounds being tested *de novo* for an ability to enhance the amino acid degrading enzyme effect to protect cells from death induced by conditions known to work via nonexcitotoxic, NO-independent mechanisms, e.g., oxidative stress, Sindbis virus infection, or staurosporine exposure, or as a secondary screen for testing compounds isolated in, for example, those assays

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described herein. Alternatively, the possible contribution of excitotoxic or NO-dependent mechanisms of cell death can be eliminated by treatment with excitatory amino acid receptor blockade, e.g., (MK-801), or 6-cyano-7-nitroqinoxaline-2,3-dione, or treatment with NO synthase inhibitors, e.g., 7-nitroindazole, N-nitro-L-arginine, or aminoguanidine.

Amino acid degrading enzymes, e.g., arginase, arginine decarboxylase, arginine deiminase, or asparaginase, and candidate compounds may be administered to animals prior to induction of neuronal death to assay for an ability to protect cells from death. Alternatively, candidate compounds may be added after induction of cell death and assessed for an ability to treat dying cells. Animal models may also serve to determine the dosage requirement for an effective compound.

Particularly useful animal models include, without limitation, Parkinson's disease (PD) rat models, which are established by injecting the catecholamine-specific neurotoxin, 6-hydroxydopamine (6-OHDA), into the medial forebrain bundle or the substantia nigra pars compact to achieve a rapid degeneration of the nigrostriatal pathway, or into the striatum to achieve progressive degeneration, as has been described (see, for example Gerlach and Riederer, J. Neural. Transm. 103: 987-1041, 1996; Bernard et al., J. Comp. Neurol. 368: 553-568, 1996; Asada et al., Ex. Neurol. 139: 173-187, 1996). Alternatively, rats may be rendered "epileptic" (i.e., induced to suffer brain seizures which often result in neuronal cell death) by administration of a variety of compounds including intravenous injection of bicuculline (Blennow et al., J. Cereb. Blood Flow Metab. 5: 439-445, 1995). Furthermore, rats may be subjected to traumatic insult to induce spinal cord injury, as described by Liu et al. (J. Neuroscience 17:5395-5406, 1997).

b) Reagents for testing in animal models

Amino acid degrading enzymes, e.g., arginine, arginine decarboxylase, arginine deiminase, or asparaginase, may optionally be administered to the central nervous

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system, e.g., intracerebrally, intracisternally, or intracerebroventricularly, of the mammal. A candidate compound is administered to the mammal via an appropriate route, e.g., parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. A candidate compound may be administered prior to the neuronal degeneration, during the neurodegeneration, immediately after the neuronal degeneration, or up to one week following neurodegeneration.

c) Results

If the candidate compound enhances the amino acid degrading enzyme protective effect against neuronal death, then the extent of neuronal death will be reduced in animals treated with the candidate compound compared to animals not treated with the candidate compound.

Therapeutics for treating neurodegeneration

1) Human neurological disorders

A number of human neurological disorders as well as neurological traumatic injury or ischemia are associated with neuronal death by a degenerative process. Amino acid degrading enzymes and compounds identified as agonists can be useful for treating, preventing, or slowing neurodegeneration, by protecting cells from nonexcitotoxic, NO-independent mechanisms of death. In particular, disorders that may be treated include, without limitation, Alzheimer's disease, Huntington's disease, Parkinson's disease, spinal muscular dystrophy, ALS, multiple sclerosis, epilepsy, or stroke.

2) Administration

Amino acid degrading enzymes, e.g., arginase, arginine decarboxylase, arginine deiminase, or asparaginase may be administered to the central nervous system by any appropriate route. For example, administration may be intracerebral, intracerebroventricular, or intracisternal.

Compounds that enhance the amino acid degrading enzyme protection against nonexcitotoxic, NO-independent mechanisms of cell death may be administered by any appropriate route. For example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for a combination of these arginine depleting enzyme-modulating reagents include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

Dosage is determined by standard techniques and is dependent, for example,

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upon the weight of the mammal treated and the type or extent of the disorder being treated.

Potentiating the therapeutic action of TNFα

1) TNFα-induced cell death

Although tumor necrosis factor (TNFα) was first named for its ability to shrink tumors, TNFα has not been effective at killing most types of cancer cells (Barinaga, Science 274:724, 1996). TNFα's cell death-inducing effect can be potentiated, however, by the coadministration of an amino acid degrading enzyme, possibly due to the enzyme inhibiting protein synthesis.

2) Administration

Amino acid degrading enzymes, e.g. arginase, arginine decarboxylase, arginine deiminase, or asparaginase, that potentiate TNFα-induced cell death may be administered by any appropriate route, as previously described (*supra*).

Other Embodiments

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is:

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Claims

1.   A method for determining whether a chemical compound affects an amino acid degrading enzyme-mediated protection from nonexcitotoxic, NO-independent cell death, said method comprising

a) contacting mammalian cells with said compound in the presence of an exogenous amino acid degrading enzyme which protects said cells against cell death, in the absence of excitotoxic stimulation and NO formation, and

b) determining the effect of said compound on the amino acid degrading enzyme-mediated protection against cell death.

2.   The method of claim 1, wherein said mammalian cells comprise neuronal cells.

3.   The method of claim 1, wherein said mammalian cells comprise glial cells.

4.   The method of claim 1, wherein said mammalian cells comprise immortalized mouse fibroblast cells.

5.   The method of claim 1, wherein said exogenous amino acid degrading enzyme is arginase.

6.   The method of claim 1, wherein said exogenous amino acid degrading enzyme is arginine decarboxylase.

7.   The method of claim 1, wherein said exogenous amino acid degrading enzyme is arginine deiminase.

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8.   The method of claim 1, wherein said exogenous amino acid degrading enzyme is asparaginase.

9.   The method of claim 1, wherein said exogenous amino acid degrading enzyme results from expression of a recombinant DNA molecule encoding the amino acid sequence of said amino acid degrading enzyme in said mammalian cells.

10.   A method for determining whether a chemical compound affects an amino acid degrading enzyme-mediated protection against nonexcitotoxic, NO-independent cell death, said method comprising

        a) contacting said compound with an amino acid degrading enzyme which protects said cells against said cell death, and

        b) determining the effect of said compound on the activity of said enzyme.   —

11.   A method for determining whether a chemical compound affects amino acid degrading enzyme-mediated protection against nonexcitotoxic, NO-independent cell death in a mammalian nervous system, said method comprising

        a) identifying a compound that affects said protection by the method of claim 1,

        b) contacting the central nervous system of a mammal with said compound, in the absence of excitotoxic stimulation or NO formation, and

        c) determining the effect of said compound on said protection against cell death in the central nervous system of said mammal.

12.   Use of a cell death protecting amount of an amino acid degrading enzyme in the preparation of a medicament for inhibiting nonexcitotoxic, NO-independent cell death in the central nervous system of a mammal.

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13. The use of claim 12, wherein said amino acid degrading enzyme is human arginase.

14. A composition for treating a human cancer patient, said composition comprising TNFα together with an amount of an amino acid degrading enzyme sufficient to potentiate TNFα-induced cell death.

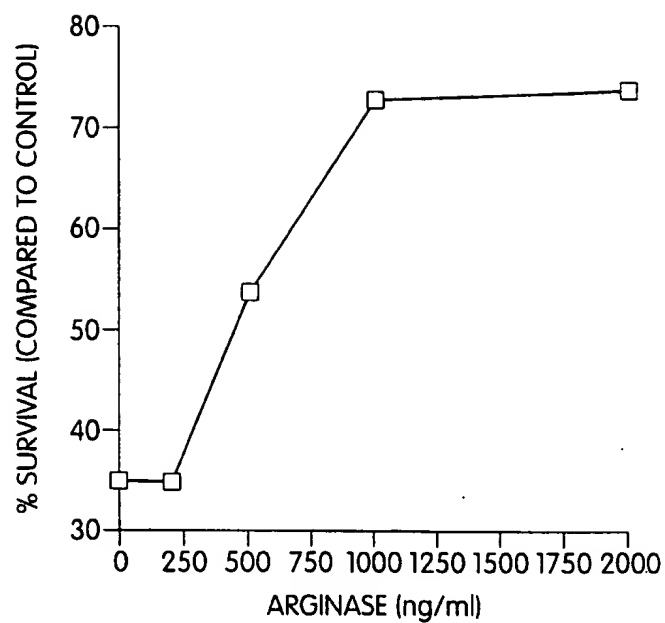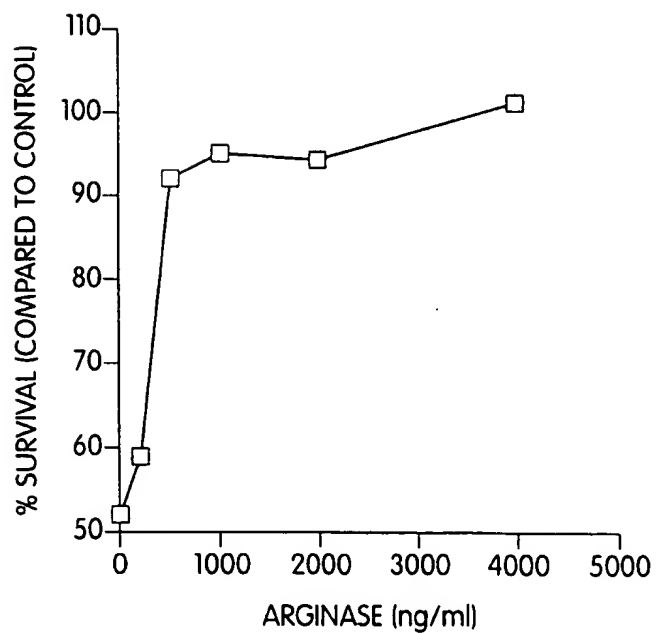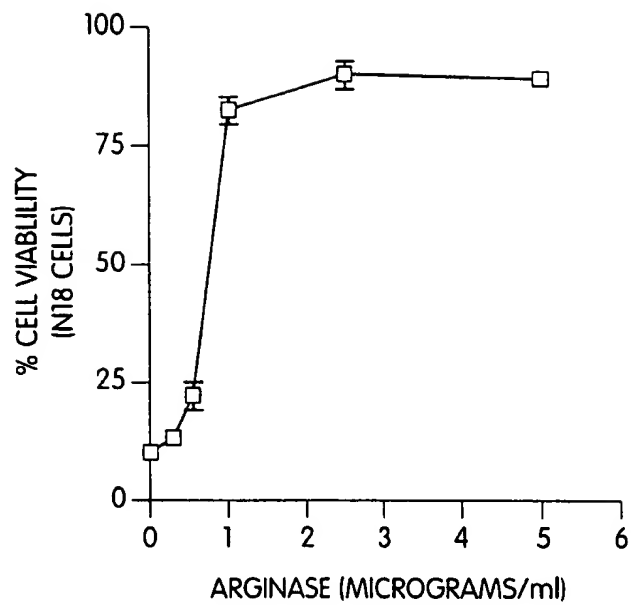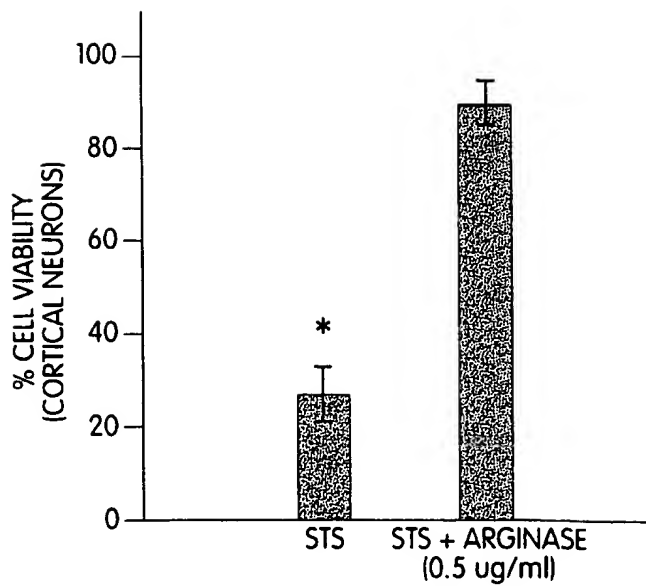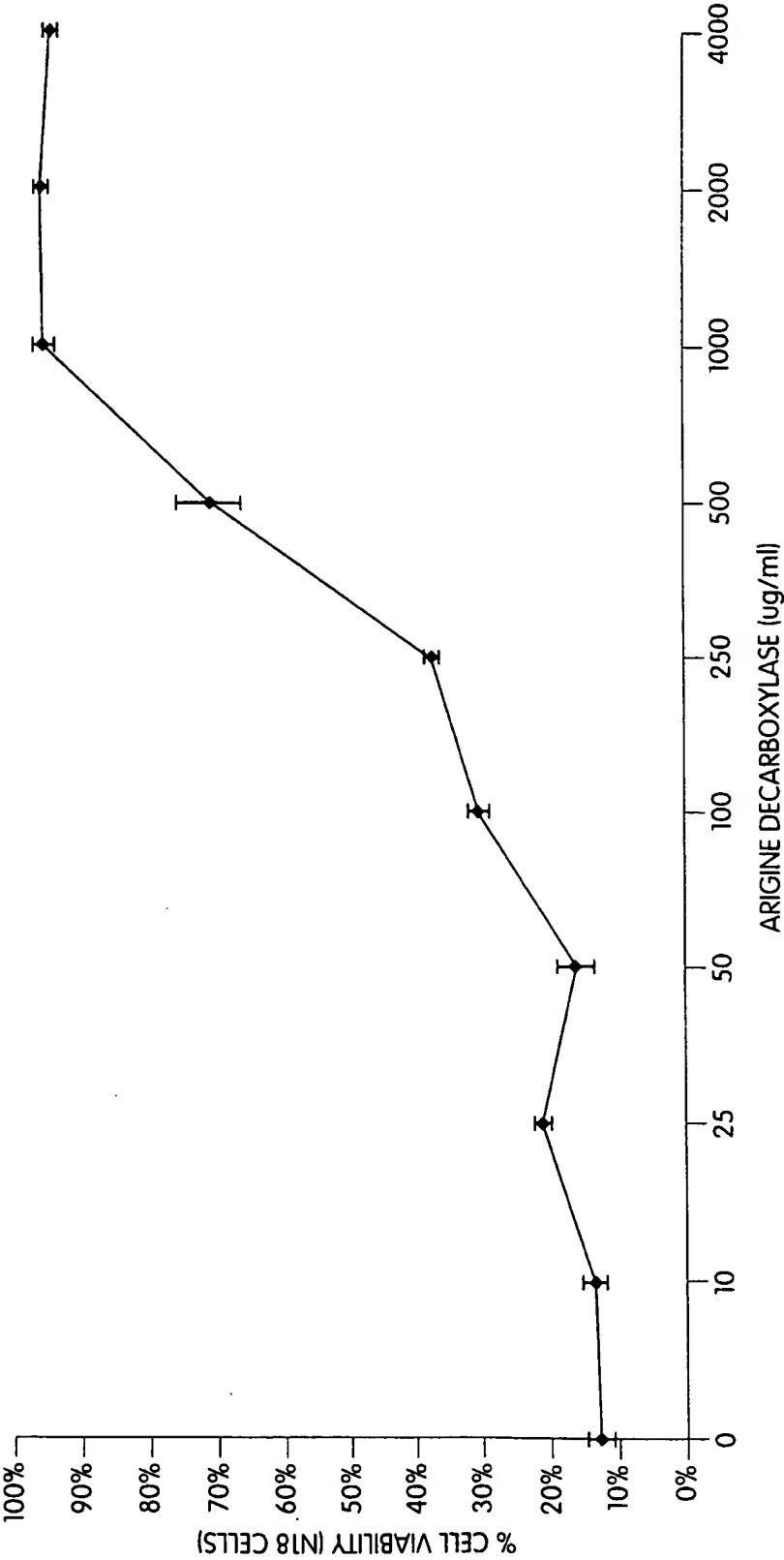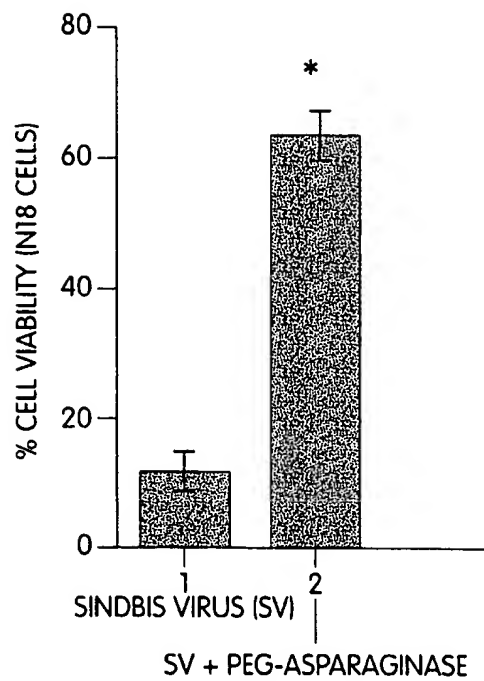15. The composition of claim 14, wherein said amino acid degrading enzyme is human arginase.

Fig. 1

Fig. 2

Fig. 2A

Fig. 3

Fig. 4

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Fig. 5
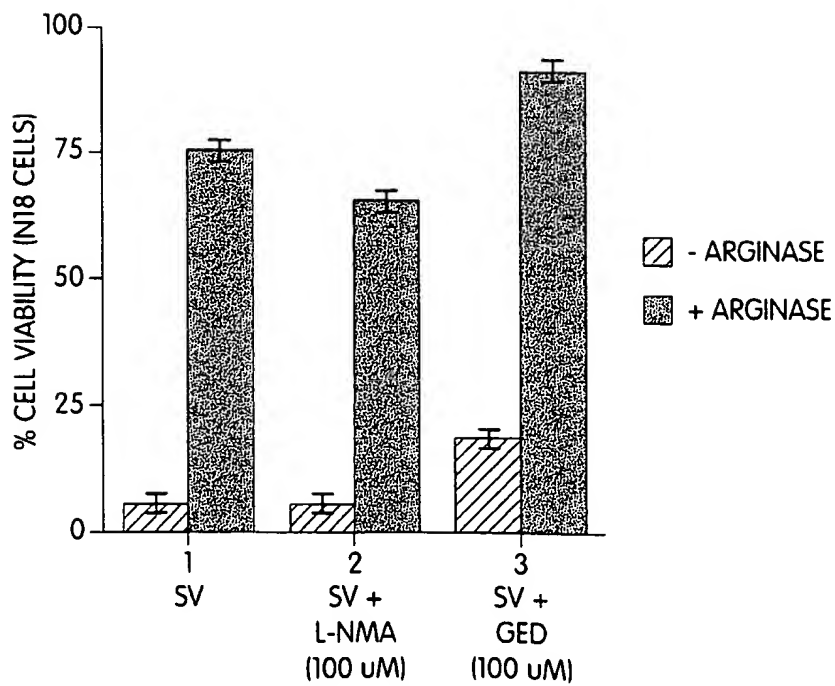
Fig. 6
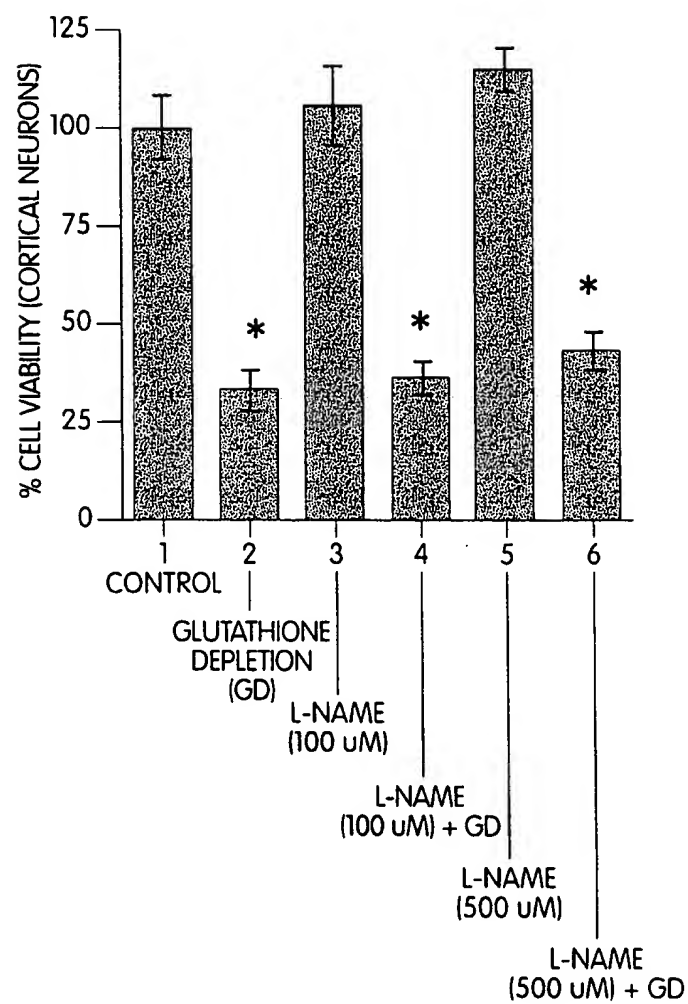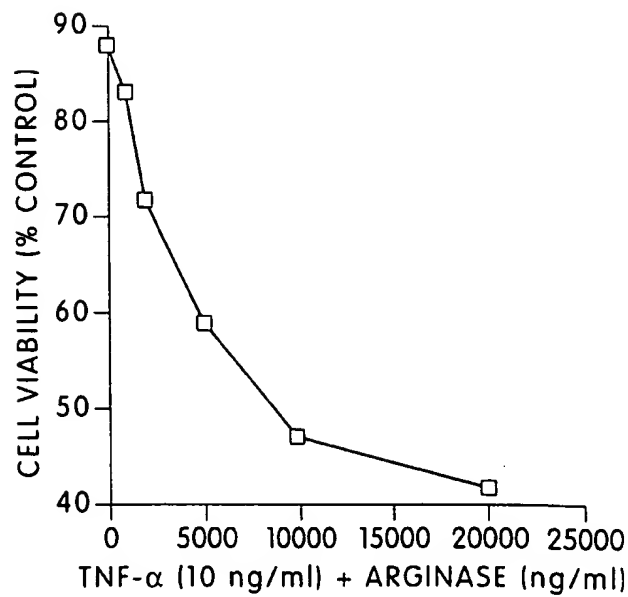
Fig. 7

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Fig. 8

Fig. 9

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/50, 49/00; C12N 9/78; C12Q 1/34
US CL   : 435/18, 227; 424/9.1, 94.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/18, 227, 375; 424/9.1, 94.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2,663,668 A (VRAT) 22 December 1953, column 4, lines 18-21, 33-49.	12-13
X	US 5,196,195 A (GRIFFITH) 23 March 1993, Examples 1-2.	12-13
X	US 5,599,984 A (BIANCHI et al.) 04 February 1997, section 5.1.3	10
P, X	ESCH et al. Purification of a Multipotent Antideath Activity from Bovine Liver and Its Identification as Arginase: Nitric Oxide-Independent Inhibition of Neuronal Apoptosis. J. Neuroscience. 01 June 1998, Volume 18, Number 11, pages 4083-4095, entire document.	1-5, 9-12

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"B"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 APRIL 1999	11 MAY 1999

Name and mailing address of the ISA/US	Authorized Officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GABRIELE ELISABETH BUGAISKY
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HRABEK et al. Comparison of Substrate and inhibitor specificity of arginase and nitric oxide (NO) synthase for arginine analogues and related compounds in murine and rat macrophages. Biochemical and Biophysical Research Communications. 14 January 1994, Volume 198, Number 1, pages 206-212, entire document.	1-5, 9-10
X	SHINOMIYA et al. Rat Liver Arginase Suppresses Mixed Lymphocyte Reaction. J. Biochemistry. 1990, Volume 107, Number 3, pages 435-439, especially final paragraph page 437.	1, 5, 9-10

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5, 9-13

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN-CAS files Registry, CAplus, Biosis, Embase Medline, APS
Search terms:arginase, modulat?, inhibit?, agoni?, antagoni?, arginine(w)(amidinase or transamidinase or
amidinohydrolase); cavanase, proclavamin? (2w)(amidino hydrolase), apopto?, antideath, fibroblast?, neuron?, glial

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single
inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees
must be paid.

Group I, claim(s)1-5 and 9-13, drawn to assay methods of altering cell death using arginase.
Group II, claim(s)1-4, 6 and 9-13, drawn to assay methods of altering cell death using arginine decarboxylase.
Group III , claim(s)1-4, 7 and 9-13, drawn to assay methods of altering cell death using arginine deiminase.
Group IV, claim(s) 1-4, 8 and 9-13, drawn to assay methods of altering cell death using asparaginase.
Group V, claims 14-15, drawn to a pharmaceutical composition of TNFα and arginase.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under
PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the technical
feature of Group I is addition of arginase inhibitors and exogenous arginase to mammalian cells in the absence of NO
synthase involvement. Hrabak *et al.* observed both *in vitro* and in long term cultures inhibition of arginase by several
specific inhibitors which had no effect on NO synthase activity.

The inventions listed as Groups I and V do not relate to a single inventive concept under PCT Rule 13.1 because, under
PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The product of
Group V differs from the product used in the method of Group I. Thus, under PCT Rules 13.1-13.2, the two Groups
lack the same technical feature.

Claims 1-4, and 9-13 of Group I will be searched only insofar as they read on a method of use of arginase.